

Short Communication

p53 codon 72 polymorphism in vulval cancer and vulval intraepithelial neoplasia

AN Rosenthal¹, A Ryan¹, D Hopster² and IJ Jacobs¹

¹Department of Gynaecological Oncology; ²Academic Department of Histopathology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary and Westfield College, West Smithfield, London EC1A 7BE, UK

Summary p53 codon 72 polymorphism was analysed in UK women with human papillomavirus (HPV)-associated vulval intraepithelial neoplasia and vulval squamous cell carcinoma. Arginine homozygotes were significantly less common in either group compared with controls. We conclude that the arginine polymorphism may confer protection against the development of HPV-associated vulval neoplasia. © 2000 Cancer Research Campaign

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Recent *in vitro* functional data (Storey et al, 1998) suggests that the arginine variant at codon 72 of the p53 tumour suppressor gene is more susceptible to degradation by oncogenic human papillomavirus (HPV) E6 protein than the proline variant. Preliminary epidemiological data (Storey et al, 1998) found that women with cervical cancer were more likely to be homozygous for arginine than healthy controls. However, several larger studies from Europe (Hayes et al, 1998; Helland et al, 1998; Joseffson et al, 1998; Lanham et al, 1998; Rosenthal et al, 1998), the USA (Hildesheim et al, 1998; Sonada et al, 1999), Japan (Wang et al, 1999) and China (Minaguchi et al, 1998; Ngan et al, 1999a) have failed to reproduce this finding. Nevertheless, one large European study (Zehbe et al, 1999) has found an excess of arginine homozygotes in cervical cancer patients from both Italy and Sweden compared with healthy controls. A weaker association was found with pre-invasive disease.

Studies of this polymorphism in the context of HPV infection have to date been limited to cervical cancer. Recent studies in head and neck cancer (Hamel et al, 2000) and skin cancer (Marshall et al, 2000) found no association between the polymorphism and malignancy, however neither study tested for HPV status, even though HPV is implicated in only a proportion of these cancers. As vulval cancer and intraepithelial neoplasia are often associated with HPV infection, we decided to investigate this polymorphism in UK women with vulval intraepithelial neoplasia (VIN) and invasive vulval squamous cell carcinoma (VSCC). While the vast majority of VIN is associated with oncogenic HPV types (Kohlberger et al, 1998), this is not the case with VSCC, in which only 15–57% of samples may contain HPV DNA (Lee et al, 1994; Kagie et al, 1997). We therefore initially tested our formalin-fixed tissue samples for the presence of genital HPV types.

MATERIALS AND METHODS

Populations studied and extraction of DNA

Samples containing both normal and neoplastic tissue (either VIN, VSCC or both) from UK caucasian women were retrieved from the pathology archives of St. Bartholomew's and the Royal London hospitals. The relevant paraffin-embedded tissue samples were serially sectioned as follows: one 4-micron section was mounted, stained with haematoxylin and eosin (H+E), covered and used as a reference slide. One 10-micron section was also stained with H+E, but left uncovered. The uncovered 10-micron section was mounted on a dissecting microscope and compared with the reference slide. Areas of tissue containing > 70% VSCC, VIN or normal tissue were identified by a histopathologist and microdissected. DNA extraction was performed in 100 µl of 10% Chelex chelating resin (Sigma, Saint Louis, MS, USA) and 200 µg ml⁻¹ proteinase K (Boehringer Mannheim, Lewes, UK), vortexed, placed in a shaking water bath at 56°C for 30 min and boiled for 8 min to inactivate the enzyme. The sample was centrifuged at 10 000 g for 10 min to pellet any remaining debris. 1–4 µl of supernatant was used directly in the polymerase chain reaction (PCR). The control population used was that described in our previous study (Rosenthal et al, 1998). Briefly, this comprised DNA extracted from 96 caucasian women from different parts of the UK taking part in a randomized controlled trial of ovarian cancer screening and 150 caucasian women attending for routine antenatal care in the Oxford region of the UK. The p53 codon 72 genotype frequencies did not differ significantly between these two groups and so they were combined for the purpose of statistical comparison with the study groups, as in our original study (Rosenthal et al, 1998).

PCR amplification of consensus genital-type HPV L1 gene

PCR in a Touchdown Thermal Cycler (Hybaid, Asford, UK) was performed in 20 µl containing 0.2 mM deoxyribonucleotide triphosphates, 0.25 U of Taq supreme DNA polymerase (Hellena

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Correspondence to: IJ Jacobs

Biosciences, Sunderland, UK), 1 × buffer (supplied with enzyme) and 20 pmol each of primers GP5+ and GP6+ (Kohlberger et al, 1998). PCR was performed using a 3 min denaturation step at 94°C followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 2 min and extension at 72°C for 1.5 min. A final extension step at 72°C for 7 min was performed. Hela cell DNA was used as positive control and cross-contamination was checked for using water controls. PCR products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide staining and ultraviolet illumination.

PCR amplification of p53 codon 72 polymorphic alleles

PCR in a Touchdown Thermal Cycler (Hybaid, Asford, UK) was performed in a volume of 20 µl containing approximately 20–100 ng DNA extracted from microdissected normal tissue, 80 µM deoxynucleotide triphosphates, 0.1 U of Red Hot DNA polymerase (Advanced Biotechnologies, Epsom, UK), 1 × buffer (supplied with enzyme), 1.5 mM Mg²⁺, 1.5 pmol of forward primer, 2 pmol of reverse primer and 0.5 pmol of ³²P-labelled forward primer. Primers for the arginine allele have been previously described (Storey et al, 1998). Primers for the proline allele were as follows: 5'-GCCAGAGGCTGCTCCCCC and 5'-GAAGGGACAGAAGATGACAGG. PCR for the arginine allele was performed using a 5 min denaturation step at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min. A final extension step at 72°C for 5 min was performed. PCR for the proline allele was performed using the same conditions as those for the arginine allele, except for a different annealing temperature of 58°C. Homozygous arginine and proline controls were used. The PCR products were electrophoresed on a 5% denaturing polyacrylamide gel. Autoradiography was performed. The arginine-specific PCR results in a 141 base-pair product and the proline-specific PCR results in a 101 base-pair product. The reliability of this assay was confirmed by direct sequencing of products amplified from the control samples from our original study (Rosenthal et al, 1998).

Statistical analysis

95% confidence intervals for the observed genotype frequencies were calculated. The chi-squared test was used to establish whether the sample and control groups were in Hardy–Weinberg equilibrium and whether the proportions of the three p53 codon 72 genotypes differed between the sample and control groups.

RESULTS

We identified 48 cases of VIN not associated with VSCC and 52 cases of VSCC which were HPV-positive. The results of the analysis are shown in Figure 1, along with the results of the control populations from our previous study (Rosenthal et al, 1998). There were significant differences in the proportions of the three p53 codon 72 genotypes (arginine homozygotes, proline homozygotes and heterozygotes) for both vulval cancer vs controls ($df = 2$, $P = 0.0023$, $\chi^2 = 12.11$) and for VIN vs controls ($df = 2$, $P < 0.0001$, $\chi^2 = 22.21$). These differences resulted from a lower frequency of arginine homozygotes in both the vulval cancer group (42%) and the VIN group (29%) compared with the control group (63%). The control population was in Hardy–Weinberg

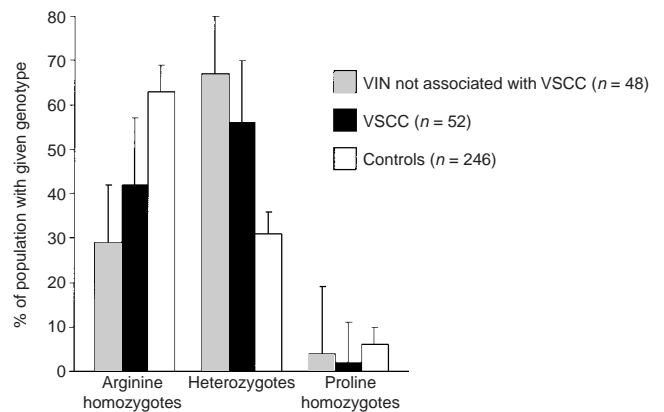


Figure 1 Distribution of p53 codon 72 alleles in women with VIN, women with VSCC and healthy controls (error bars = 95% confidence intervals)

equilibrium ($df = 1$, $P = 0.123$, $\chi^2 = 2.38$), but neither the vulval cancer nor the VIN populations were in equilibrium (vulval cancer $df = 1$, $P = 0.016$, $\chi^2 = 5.76$; VIN $df = 1$, $P = 0.003$, $\chi^2 = 8.56$).

DISCUSSION

If women homozygous for the arginine variant of the p53 codon 72 polymorphism are at increased risk of cervical cancer (Storey et al, 1998), then this has important implications for targeting cervical screening to a high-risk population. As the polymorphism was thought to increase cervical cancer risk by rendering p53 product more susceptible to HPV E6-mediated degradation, the arginine variant might also be expected to increase the risk of other anogenital HPV-associated neoplasias. Included in this group are VIN and some VSCC, in which the predominant HPV is the oncogenic type 16 (Hording et al, 1993; Lee et al, 1994; Kagie et al, 1997; Ngan et al, 1999b). We therefore tested the hypothesis that HPV-positive VIN and VSCC were associated with being homozygous for the arginine variant. If such an association were found, such women might benefit from increased surveillance. This study is the first to examine the relationship between p53 codon 72 polymorphism and proven HPV-associated neoplasia outside the cervix.

We demonstrated a significant association between the p53 codon 72 polymorphism genotype and the type of sample studied. However, in contrast to the two previous positive studies in cervical cancer (Storey et al, 1998; Zehbe et al, 1999), we observed a lower frequency of arginine homozygotes in the HPV-associated vulval cancer and VIN patients than in healthy controls. This suggests that arginine homozygotes have a lower risk of developing vulval neoplasia, compared with women who have one or more proline alleles. This is supported by the observation that neither the VSCC nor the VIN population were in Hardy–Weinberg equilibrium, indicating that the presence of a proline allele appears to select in favour of the vulval neoplasia phenotype. We are unaware of any biologically plausible reason as to why the arginine variant might confer protection, but it is interesting to note that studies of lung (Birgander et al, 1995) and nasopharyngeal cancer (Birgander et al, 1996) have also demonstrated an association between the proline allele and disease. However, the risk of the proline allele applied mostly in combination with the presence of a 16 base-pair duplication in intron 3 of the p53 gene. This suggests that the proline allele may be a marker

for linkage disequilibrium with a functional cancer susceptibility site elsewhere in the gene.

Eleven of 13 different populations reported in the 11 studies so far published have failed to demonstrate an association between p53 codon 72 polymorphism and cervical cancer, while two have found a positive association between this disease and the arginine variant. There are three possible explanations for the conflicting results in these studies. First, accidental inclusion of HPV-negative cancers in some of the studies could have diluted the true study population. This criticism is not relevant in cervical cancer where almost all squamous cell carcinomas (SCC) are associated with HPV (Walboomers et al, 1999). It could be argued that of the oncogenic HPV types, only HPV 16 and 18 E6 proteins have been shown to degrade the arginine variant of p53 more easily than the proline variant (Storey et al, 1998) and that other types of oncogenic HPV may not exhibit this property. However approximately 75% of cervical SCC in Europe contain these two types of HPV (Bosch et al, 1995), so this is an unlikely explanation. In the case of vulval cancer, not all cases are HPV-positive, so we used sensitive consensus primers to detect genital HPV types and only performed the polymorphism analysis in HPV-positive cancers. Previous studies (Hording et al, 1993; Lee et al, 1994; Kagie et al, 1997; Ngan et al, 1999b) have demonstrated that the vast majority of HPV-positive VSCC contains type 16.

A second possible explanation for discrepant results in polymorphism studies is accidental inclusion of people from different ethnic groups. We were careful to ensure that only UK caucasian women were included in both our previous study and this study. The other studies of this polymorphism describe ethnic matching of cases and controls. It is interesting to note that the UK caucasian control population from the original pilot study (Storey et al, 1998) had different allele frequencies to the larger UK caucasian populations in subsequent studies (Rosenthal et al, 1998; Lanham et al, 1998). While the pilot study control population allele frequencies may not have been statistically in disequilibrium, this may reflect the relatively small sample size.

The third possible explanation for discrepant results is the accuracy of the polymorphism assay used. While we cannot comment on different methods used by other groups, our previous report (Rosenthal et al, 1998) used the same technique as that used in the pilot study (Storey et al, 1998). We modified this technique in the current study because the frequency of loss of heterozygosity at the p53 locus in VSCC or VIN has not yet been reported. It is therefore possible that performing the polymorphism assay on samples containing neoplastic tissue could underestimate the proportion of true heterozygotes if some of them had lost one copy of the p53 locus. We therefore tested microdissected normal tissue. It was necessary to redesign one of the primers for amplification of the proline allele in order to yield a relatively smaller product, which could be reliably amplified from the lower concentrations of DNA obtained from microdissected tissue. Direct sequencing of this product confirmed that it amplified the proline variant.

In conclusion, the balance of evidence suggests that the arginine polymorphism, while functionally different from the proline in vitro, does not appear to be associated with an increased risk of cervical neoplasia. Even if much larger studies were to refute this conclusion, the high frequency of arginine homozygotes in Northern European populations means that this polymorphism is unlikely to be useful as a test to identify women at higher risk of

cervical cancer in such populations. In contrast to the evidence in cervical cancer, our data suggests that the arginine variant may protect against the development of HPV-associated vulval neoplasia.

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